Research Article

Coactivation of Liver Receptor Homologue-1 by Peroxisome Proliferator-Activated Receptor γ Coactivator-1α on Aromatase Promoter II and Its Inhibition by Activated Retinoid X Receptor Suggest a Novel Target for Breast-Specific Antiestrogen Therapy

Rachid Safi,[1] Agnes Kovacic,[2,3] Stéphanie Gaillard,[1] Yoko Murata,[2] Evan R. Simpson,[2,3] Donald P. McDonnell,[1] and Colin D. Clyne[2,3]

[1]Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina; [2]Prince Henry's Institute of Medical Research; and [3]Department of Biochemistry, Monash University, Clayton, Victoria, Australia

Abstract

Aromatase inhibitors target the production of estrogen in breast adipose tissue, but in doing so, also decrease estrogen formation in bone and other sites, giving rise to deleterious side effects, such as bone loss and arthralgia. Thus, it would be clinically useful to selectively inhibit aromatase production in breast. In this regard, we have determined that the orphan nuclear receptor liver receptor homologue-1 (LRH-1) is a specific transcriptional activator of aromatase gene expression in human breast preadipocytes but not in other tissues of postmenopausal women. In this study, we show that the coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) is a physiologically relevant modulator of LRH-1, and that its transcriptional activity can be inhibited effectively using receptor-interacting peptide antagonists that prevent PGC-1α recruitment. Interestingly, we note that all of these peptides also interact in an agonist-dependent manner with retinoid X receptor α (RXRα), suggesting that these two receptors may compete for limiting cofactors within target cells. In support of this hypothesis, we show that 9-*cis*-retinoic acid, acting through RXR, inhibits both the basal and PGC-1α–induced transcriptional activity of LRH-1. The importance of this finding was confirmed by showing that LRH-1–dependent, PGC-1α–stimulated regulation of aromatase gene expression in primary human breast preadipocytes was effectively suppressed by RXR agonists. We infer from these data that LRH-1 is a bona fide target whose inhibition would selectively block aromatase expression in breast, while sparing other sites of expression. (Cancer Res 2005; 65(24): 11762-70)

Introduction

The orphan nuclear receptors liver receptor homologue-1 (LRH-1, CPF, FTF, NR5A2) and steroidogenic factor-1 (SF-1, AD4BP, NR5A1) are the two human homologues of the *Drosophila* nuclear receptor FTZ-F1 (1). SF-1 expression is mainly restricted to steroidogenic tissues, such as the adrenal cortex, and to those of the reproductive axis, where it regulates the expression of genes involved in steroid homeostasis (2). LRH-1 was initially described to be expressed in the liver, pancreas, and intestine, where it governs the expression of transcription factors involved in hepatic and pancreatic differentiation. In addition, LRH-1 has been shown to be a primary regulator of cholesterol homeostasis, where among other things, it has shown to be involved in the regulation of the expression of *CYP7A1*, the rate-limiting enzyme in the classic pathway of bile acid biosynthesis, and *CYP8B1*, a gene involved in cholic acid synthesis (reviewed in ref. 3). Recently, LRH-1 was shown to be expressed in the ovary and testis, where it has been suggested that it may play a role in steroid hormone biosynthesis (4–6), although this latter hypothesis remains controversial (4). Regardless, the recent demonstration that LRH-1, but not SF-1, is coexpressed in mesenchymal preadipocytes with aromatase suggests that this receptor may be a physiologically relevant regulator of estrogen biosynthesis (7). Interestingly, we have also shown that LRH-1 can bind and activate aromatase promoter II and is coexpressed with aromatase in breast preadipocytes, and furthermore, that aromatase expression is high in breast cancers, where LRH-1 is expressed (7, 8). Significantly, in the presence of a tumor, aromatase expression in breast preadipocytes is increased due to the activation of the gonadal promoter II and the overlapping promoter I.3 (9), and it seems likely that LRH-1 is the key transcription factor responsible for this enhancement of aromatase expression (7, 8).

For the ligand-dependent nuclear receptors, binding of agonists induces an active conformational change within the receptor, resulting in the repositioning of helix-12, which facilitates transcriptional coactivator recruitment (10). Whether or not activation of LRH-1 is ligand dependent is unclear. The crystal structure of mouse LRH-1 revealed a large, empty ligand-binding cavity (11), but that of the human LRH-1-LBD showed the presence of bacterial phospholipids (12–14). However, the role of phospholipids in regulating the activity of LRH-1 remains to be established, and thus the nature and abundance of corepressors and coactivators seems to be the primary determinant of LRH-1 activity.

In this study, we show that the coactivators glucocorticoid receptor-interacting protein 1 (GRIP1) and peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) both interact with and enhance LRH-1 transcriptional activity and thus are serving as "protein ligands" for this receptor. These coactivators are likely to be of physiological importance, as peptide antagonists that prevent their interaction with LRH-1 effectively inhibit the activity of this receptor at target gene promoters. This suggests that in addition to LRH-1 expression, the relative and absolute expression levels of the coactivators GRIP1 and PGC-1α will influence aromatase expression in breast adipose tissue. Exploiting this observation, we have shown that agonist-activated retinoid X

Requests for reprints: Donald P. McDonnell, Department of Pharmacology and Cancer Biology, Duke University Medical Center, Box 3813, Durham, NC 27710. Phone: 919-684-6035; Fax: 919-681-7139; E-mail: Donald.McDonnell@duke.edu.

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receptor α (RXRα) can also block LRH-1 activity by competing for the binding of these limiting coactivators, and thus not surprisingly, RXR agonists can inhibit LRH-1/PGC-1α–dependent regulation of the aromatase gene expression in primary human preadipocytes.

Materials and Methods

Expression constructs. Human LRH-1 (Genbank database gi: 2007016; −16 and 1,515 bp) was generated by reverse transcription-PCR from human liver polyadenylated mRNA (Clontech, Palo Alto, CA). The resulting cDNA was cloned into pCMVtag (Stratagene, La Jolla, CA) or pVP16 expression vectors (Clontech). To produce a Gal4-LRH-1 LBD fusion construct, the region from 228 to 1,515 bp was amplified by PCR from pCMVtag LRH-1 and cloned to pM (Gal4-DBD; Stratagene). pCMX expression plasmid for mouse LRH-1 was provided by Dr. D.J. Mangelsdorf (The University of Texas Southwestern Medical Center, Dallas, TX). RST7 expression plasmid for human RXRα was provided by Ligand Pharmaceuticals (San Diego, CA). pcDNA 3.1 expression vector for PGC-1α was provided by Dr. B.M. Spiegelman (Dana-Farber, Boston, MA). pCMX-GRIP1 was provided by Dr. M.R. Stallcup (University of Southern California, LA, CA). PGC-1α and GRIP1 NR box mutants were generated by mutating the wild-type LXXLL within the NR box to AXXAA by PCR-directed mutagenesis using the QuickChange Site-Directed mutagenesis kit (Stratagene) following the protocol provided by the manufacturer. pcDNA.3 expression vector for PGC-1β was provided by Dr. C.M. Newgard (Duke University Medical Center, Durham, NC). PII-516 is a *CYP19* promoter II/luciferase construct containing −516/−17 nucleotides of the human *CYP19* promoter II as described (7).

Cell culture, transfection, and reporter gene assays. HeLa and HepG2 cells were cultured in MEM Modified Eagle Medium (Life Technologies Bethesda Research Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate (Life Technologies Bethesda Research Laboratories) and maintained in a humidified 37°C incubator with 5% CO_2. The cells were seeded overnight into 24-well cell culture plates. The cells were transfected with 1.5 μg of luciferase reporter, 0.1 μg of cytomegalovirus β-galactosidase (pCMVβgal) per triplicate using FuGENE 6 reagent (Roche, Indianapolis, IN). The amounts of expression plasmids per transfection were 100 ng wild-type LRH-1 or empty vector. For coactivation assays, a dose from 0 to 400 ng of pCMX-GRIP1 was used and 0 to 200 ng for pcDNA-3.1 PGC-1α/β. When the mutants were used, 250 ng of pCMX-GRIP1 wild-type or NR box mutants were transfected in the presence of LRH-1 expression vector or an empty vector. For PGC-1α NR box mutants, 100 ng expression plasmids were used. In mammalian two-hybrid assays, for a typical triplicate of transfection, 1,500 ng of 5xGal4Luc3 reporter plasmid, 400 ng of receptor-VP16 fusion, 400 ng of pM (Gal4DBD)-NR box fusion constructs, and 100 ng of normalization plasmid pCMVβgal were used. NIH-3T3 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with PII-516/luciferase reporter, 100 ng of pCMVβgal, and 100 ng of mouse LRH-1 expression construct (or empty vector). Cells were serum starved for 24 hours before treatment with 25 μmol/L of forskolin and 4 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma, St Louis, MO). Following a 20-hour transfection, medium was replaced with fresh media. Cells were lysed 36 to 40 hours after transfection and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity to correct for transfection efficiency. All transfections were done in triplicate. The results presented were typical of several independent transfection experiments. Ligands were dissolved in ethanol or DMSO before use in cell culture. LG100268, LG101506, and LG101208 were generous gifts of Ligand Pharmaceuticals.

Affinity selection of LRH-1-interacting peptides. Human LRH-1 was cloned into the *Eco*RI sites of a baculovirus shuttle vector pDW464 to make an in-frame fusion of the LRH-1 with the biotin acceptor peptide (Science Reagents, El Cajon, CA). Recombinant LRH-1 baculovirus was generated using Bac-To-BacR Baculovirus Expression System (Life Technologies Bethesda Research Laboratories) following the protocol provided by the manufacturer. LRH-1 recombinant protein was produced in Sf9 insect cells following infection with recombinant baculovirus particles. A soluble extract of infected Sf9 cells was used to affinity purify biotinylated LRH-1 fusion protein with monomeric avidin resin (Promega Corp., Madison, WI). To select for LRH-1 binding peptides, a modified protocol from that previously described (15) was used. Briefly, ~2 μg of baculovirus-expressed full-length LRH-1 protein were diluted in 100 μL of PBST [137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na_2HPO_4 (pH 7.3), 0.1% Tween 20] and applied to a single well in a DNA-coated cell culture plate. To prepare the DNA-coated plate, a 96-well Costar plate was first coated with 20 μg of Neutravidin Biotin-Binding Protein (Pierce Biotechnology, Rockford, IL) overnight at 4°C, and 2 pmol of double-stranded 5′-biotinylated oligonucleotides (diluted in PBST) containing an SF-1 response element (SFRE) were added for 1 hour at room temperature. The protein-coated plate was incubated overnight at 4°C. The wells were then blocked with 150 μL of 2% milk in $NaHCO_3$ (pH 8.5) for an additional 1 hour at room temperature and washed five times with PBST to remove unbound proteins. Then, 25 μL of the phage peptide library (with 10^{10} phage particles) diluted in 100 μL of MPBS (2% nonfat dry milk in PBS) was added to the wells, and the plate was sealed and incubated for 3 hours at room temperature. Construction of the LXXLL M13-phage library was described previously (15). Nonbinding phage were removed by washing the wells five times with 300 μL of PBST. The bound phage were eluted with 100 μL of 0.1 mol/L HCl for 10 minutes. The eluent was neutralized with 50 μL of 1 mol/L Tris-HCl (pH. 7.4). Phage eluted from the target were amplified in *Escherichia coli* DH5αF′ cells for 5 hours at 37°C, and the supernatant containing amplified phage was collected for use in subsequent rounds of panning; a total of four rounds of panning were done. A PCR reaction was then done using 1 μL of the unamplified eluent phage from both rounds 3 and 4, with mBax reverse and forward primers to amplify peptide inserts. PCR products were purified and digested with *Xba*I and *Xho*I before their cloning in a modified pM (Gal4-DBD) vector. The peptide sequences were deduced by DNA sequencing.

Primary human adipose stromal cells. Human adipose stromal cells were isolated and cultured as described previously (16). Once confluent, cells were serum starved for 24 hours before treatment with experimental agents for a further 24 hours. The method of adipocyte differentiation has also been described previously (7).

Real-time PCR. Real-time PCR quantification of mRNA expression of aromatase and LRH-1 has been described previously (7). Primers used for amplification of LRH-1, aromatase promoter II, and PGC-1 isoforms were (5′-3′) as follows: LRH-1 (sense, CTGATACTGGAACTTTTGAA; antisense, CTTCATTTGGTCATCAACCTT), aromatase pII (sense, TTGGAAATGCT-GAACCCGAT; antisense, CAGGAATCTGCCGTGGGAGA), PGC-1α (sense, TCAGTCCTCACTGGTGGACA; antisense, TGCTTCGTCGTCAAAAACAG), PGC-1β (sense, TGTTCAGACAGAACGCCAAG; antisense, ACACCGGTAGT-GATGAAGC).

Results

GRIP1 and PGC-1α function as transcriptional coactivators for LRH-1. The ability of the ubiquitously expressed coactivator GRIP1/TIF2/SRC-2 to regulate LRH-1 transcriptional activity was examined using transient transfection assays in HeLa cells. Specifically, an aromatase promoter II-luciferase reporter construct was transfected into cells in the absence or presence of coexpressed LRH-1 and increasing amounts of a GRIP1 expression vector. As expected, cotransfection of LRH-1 resulted in a strong activation (10-fold) of the aromatase promoter II (Fig. 1*A*). Coexpression of GRIP1 significantly enhanced LRH-1-mediated transcription, up to 6-fold, at the maximal concentration of expression plasmid tested. To verify that this effect was not limited specifically to the aromatase promoter II, we repeated this experiment using another LRH-1-regulated promoter, that of the small heterodimer partner (SHP; ref. 17), and obtained similar results (data not shown).

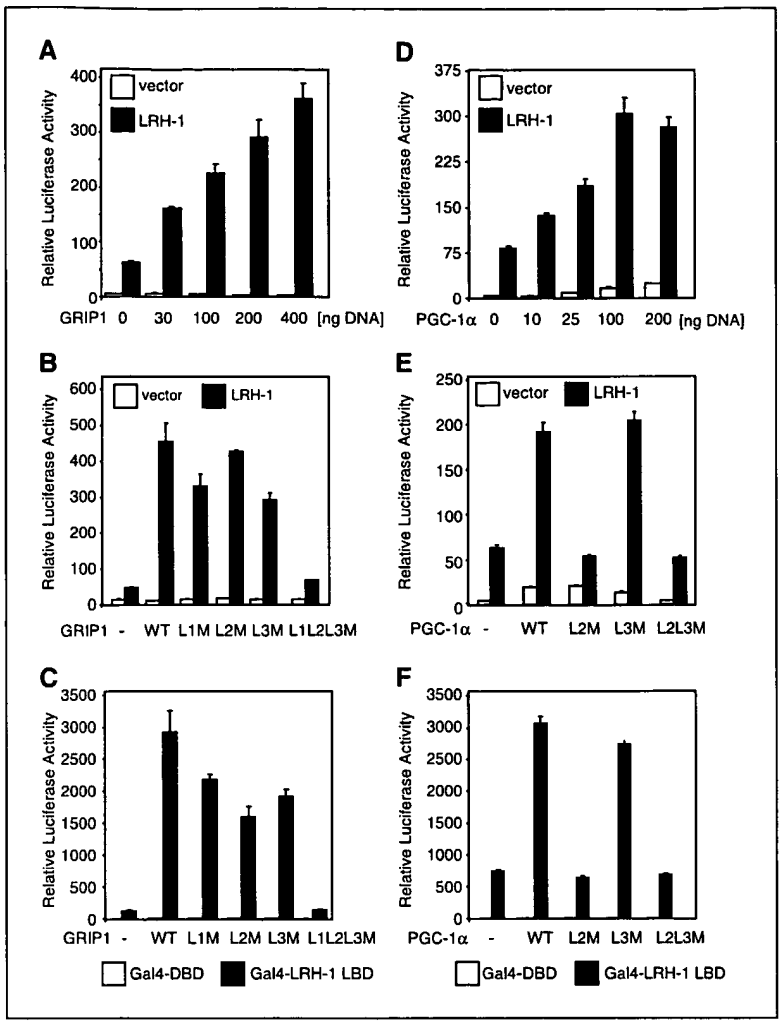

Figure 1. GRIP1 and PGC-1α coactivate LRH-1 transcriptional activity. *A*, GRIP1 functions as a coactivator for LRH-1. HeLa cells were transfected with an aromatase promoter II-luciferase (PII-516) construct in the presence (*solid columns*) or absence (*open columns*) of an LRH-1 expression vector and increasing concentrations of a GRIP1 expression construct (0-400 ng). Relative luciferase activity is indicated. *B*, GRIP1 coactivates LRH-1 through its LXXLL motifs. HeLa cells were transfected with an aromatase promoter II-luciferase construct in the presence (*solid columns*) or absence (*open columns*) of an LRH-1 expression vector and various GRIP1 expression constructs encoding either the wild-type protein (*WT*), or protein harboring mutations either in each individual NR box (L1M, L2M, and L3M), or in all three NR boxes together (L1L2L3M). Relative luciferase activity is indicated. *C*, GRIP1 directly interacts with LRH-1 in mammalian cells. HeLa cells were transfected with the GRIP1 NR box mutants described above, in the presence of a Gal4-LRH-1 LBD expression vector, and a GAL4-responsive luciferase reporter gene. Relative luciferase activity is indicated. *D*, PGC-1α stimulates LRH-1-mediated transcription. HeLa cells were transfected with an aromatase promoter II-luciferase construct in the presence (*solid columns*) or absence (*open columns*) of an LRH-1 expression vector and increasing concentrations of a PGC-1α expression construct (0-200 ng). Relative luciferase activity is indicated. *E*, PGC-1α coactivation of LRH-1 requires intact LXXLL motif within NR box 2. HeLa cells were transfected with an aromatase promoter II-luciferase construct in the presence (*solid columns*) or absence (*open columns*) of an LRH-1 expression vector and various PGC-1α expression constructs encoding either the wild-type protein (*WT*), or protein harboring mutations in either each individual NR box (L2M and L3M), or both NR boxes together (L2L3M). Relative luciferase activity is indicated. *F*, LRH-1 interacts with the NR box 2 of PGC-1α. HeLa cells were transfected with the PGC-1α NR box mutants described above, in the presence of a Gal4-LRH-1 LBD expression vector, and a Gal4-responsive luciferase reporter gene. Relative luciferase activity is indicated.

It is well documented that the p160 family of coactivators interact with the nuclear receptors using a specific LXXLL-containing domain, the NR box. Thus, a series of GRIP1 NR box mutants were created, in which each LXXLL motif was disrupted individually or in combination. Specifically, LXXLL in each NR box was mutated to AXXAA, yielding the mutants L1M, L2M, and L3M. A triple mutant (L1L2L3M), where all three NR boxes are mutated within the context of full-length GRIP1, was also created. As shown in Fig. 1*B*, mutation of all three motifs completely abrogated GRIP1-dependent stimulation of LRH-1 transcriptional activity. GRIP1 constructs, in which only a single NR box was mutated, exhibited a significant GRIP1-dependent stimulation of LRH-1 transcriptional activity, whereas the L1M and L3M mutants were slightly less active than wild type (Fig. 1*B*). To rule out the possibility that the effect seen with GRIP1 was not due to effects on other transcription factors that bound the aromatase promoter, we evaluated the effect of GRIP1 expression on the activity of a Gal4-LRH-1 LBD chimera using a Gal4-responsive luciferase reporter. As seen in Fig. 1*C*, Gal4-LRH-1 LBD has minimal intrinsic transcriptional activity that is dramatically enhanced by the expression of GRIP1. Analysis of the GRIP1 mutants in this assay gave similar results as LRH-1 assayed on the aromatase promoter II (Fig. 1*C*). Thus, GRIP1-dependent stimulation of LRH-1 transcriptional activity is likely to occur as a consequence of a direct interaction between these two proteins.

Although many nuclear receptor coactivators are ubiquitously expressed, a tissue-specific coactivator, PGC-1α, has been shown to be preferentially expressed in adipose tissue, liver, and muscle (18, 19). Although originally identified as a PPARγ-specific coactivator, PGC-1α has since been shown to coactivate several other nuclear receptors (20). We therefore asked whether PGC-1α can function as a coactivator for LRH-1. To this end, HeLa cells were transfected with an aromatase promoter II reporter gene construct in the absence or presence of LRH-1 and increasing amounts of PGC-1α. In this manner, we showed that PGC-1α is a robust coactivator of LRH-1 transcriptional activity (Fig. 1*D*).

PGC-1α contains one consensus LXXLL sequence (L2) that has been shown to be the major binding site for many nuclear receptors. A mutation in L2 (LXXLL to AXXAA) abolished PGC-1α–mediated stimulation of LRH-1 transcriptional activity, whereas a mutation in the third leucine-rich motif (L3) had no effect (Fig. 1*E*). Not surprisingly, mutation of both motifs (L2L3M) abolished PGC-1α–dependent coactivation completely. Notably, these PGC-1α mutants had similar effects on LRH-1 transactivation in the context of a Gal4-LRH-1 fusion protein (Fig. 1*F*) as they did on the aromatase promoter II (Fig. 1*E*), suggesting that L2 is both necessary and sufficient for PGC-1α recruitment by LRH-1. We did similar experiments using PGC-1β and found that this closely related cofactor had no effect on either LRH-1-induced aromatase promoter II activity or Gal4-LRH-1 transcriptional activity (data not shown).

We conclude from these series of experiments that both the ubiquitously expressed GRIP1 and the tissue-restricted PGC-1α (but not PGC-1β) can act as coactivators for LRH-1.

PGC-1 expression in preadipocytes. We next asked whether PGC-1α is a physiologically relevant coactivator of LRH-1, with respect to the regulation of aromatase gene expression in preadipocytes. Aromatase is expressed in undifferentiated primary human breast preadipocytes rather than in mature adipocytes, and we have previously shown that stimulation of aromatase gene transcription by LRH-1 is dramatically elevated in the presence of cyclic AMP (cAMP), an inducer of PGC-1α expression (21). To determine whether PGC-1α is coexpressed with LRH-1 in preadipocytes and regulated by cAMP, we quantified by real-time PCR the mRNA expression of PGC-1α, PGC-1β, LRH-1, and aromatase in primary human preadipocytes treated with or without forskolin (to increase intracellular cAMP), or in cells that had been differentiated into mature adipocytes by incubation in an adipogenic medium for 14 days (Fig. 2). PGC-1α mRNA levels in preadipocytes were markedly stimulated by forskolin (~17-fold), and no difference in PGC-1α expression was evident between untreated preadipocytes and differentiated adipocytes (Fig. 2*A*). In contrast, PGC-1β mRNA levels were ~10-fold lower than PGC-1α mRNA in resting preadipocytes, and although forskolin increased PGC-1β mRNA levels by ~7-fold, the greatest increase in PGC-1β mRNA occurred in differentiated adipocytes (20-fold; Fig. 2*B*). LRH-1 mRNA was increased by ~2-fold upon forskolin treatment of preadipocytes and was undetectable in differentiated adipocytes (Fig. 2*C*). Aromatase mRNA levels in preadipocytes were strongly induced by forskolin (~10-fold) and, consistent with previous studies (7), significantly lower in differentiated adipocytes than in preadipocytes (Fig. 2*D*). Therefore, aromatase, PGC-1α, and LRH-1 are coexpressed in preadipocytes and positively regulated by cAMP. Expression of PGC-1β, which does not coregulate LRH-1 or stimulate aromatase expression, is associated with the differentiated adipocyte rather than the undifferentiated preadipocyte phenotype.

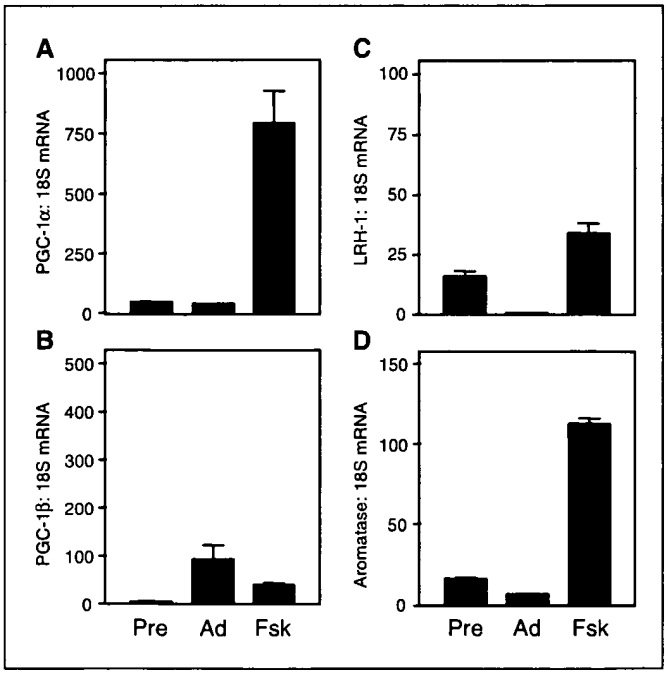

Figure 2. PGC-1α is expressed in human preadipocytes and is positively regulated by cAMP. Primary cultures of human preadipocytes (*Pre*) were either differentiated for 12 days in adipogenic medium (*Ad*), or treated for 24 hours with forskolin (*Fsk*). Total RNA was extracted and quantitative real-time PCR was done using primers specific for (*A*) PGC1α, (*B*) PGC-1β, (*C*) LRH-1, and (*D*) *CYP19*. *Columns*, means of triplicate determinations (fg target cDNA/μg RNA) from one representative experiment; *bars*, SD. Similar results were obtained in two further independent experiments.

Development of peptide antagonists that target LRH-1/coactivator interactions. To date, no chemical ligands have been developed that can modulate LRH-1 transcriptional activity. However, given the importance of the coactivators GRIP1 and PGC-1α for LRH-1 transcriptional activity and that their interaction with LRH-1 occurs through a specific short LXXLL sequence, we reasoned that it might be possible to develop peptide antagonists that function by specifically blocking LRH-1/coactivator interactions. To develop a peptide-screening assay, recombinant full-length LRH-1 was purified from baculovirus-infected Sf9 cells and immobilized on a plastic-bound SFRE oligonucleotide. This system was used for *in vitro* selection of an M13 phage library expressing short 19-mer peptides in the format $(X)_7LXXLL(X)_7$ (15). We previously determined that the diversity in the library is sufficient for identification of peptides, which display the affinity and specificity needed to be useful as antagonists of NR function in intact cells. Figure 3*A* shows representative peptide sequences derived from phage isolated following four successive rounds of screening. In spite of the complexity of the random phage library (exceeding 10^8 independent clones), clustal peptide sequence alignment of LRH-1-binding peptides shows the presence of a strong consensus sequence motif PILRXLL (Fig. 3*A*). Further analysis of peptide sequences revealed that the sequence of the peptide L4-1 is homologous to the third NR box of Dax-1, whereas the L3-20 peptide is more closely related to the second NR box in SHP. These two proteins are known to bind and inhibit LRH-1-mediated transcriptional activity (22–24). Peptides with these sequence characteristics have previously been described as "class III-like LXXLL peptides." Interestingly, such sequences constitute the NR box regions of Dax-1, SHP, or PGC-1α (15).

We next assessed the ability of these peptides to interact with LRH-1 in intact cells using a mammalian two-hybrid assay. For this purpose, full-length LRH-1 was expressed as a fusion protein with the VP16 acidic activation domain, and the peptides were produced as fusion proteins with the yeast Gal4-DBD. Interaction between LRH-1-VP16 and the LXXLL peptides-Gal4-DBD fusions was assessed by using the 5xGal4Luc3 luciferase reporter gene. Figure 3*B* shows the interaction of the peptides with LRH-1. Although some of the peptides interacted with LRH-1 better than others, all of them bound with a greater efficacy to VP16-LRH-1 than to the VP16 empty vector control: between 2-fold (peptide L3-20) and 6-fold (peptide L4-16; Fig. 3*B*). As expected, a construct containing the isolated LRH-1-LBD fused to the Gal4-DBD confirmed that these peptides interact with LRH-1 in an LBD-dependent manner (data not shown). We also observed that some peptides have a low but significant basal level of interaction in the absence of VP16-LRH-1, due most likely to their interaction with endogenous LRH-1 in HepG2 cells. We next assessed the ability of the peptide-Gal4 fusions to inhibit LRH-1 transcriptional activity. Coexpression of peptides L3-11, L3-12, L4-1, or L4-16 with an LRH-1 expression vector and luciferase reporter vector driven by five copies of the SFRE showed that these peptides repressed LRH-1-mediated transcription in a dose-dependent manner, reaching 100% inhibition at doses between 0.16 and 0.33 μg of the vector expressing the Gal4-peptide fusion (Fig. 3*C*). These data suggest

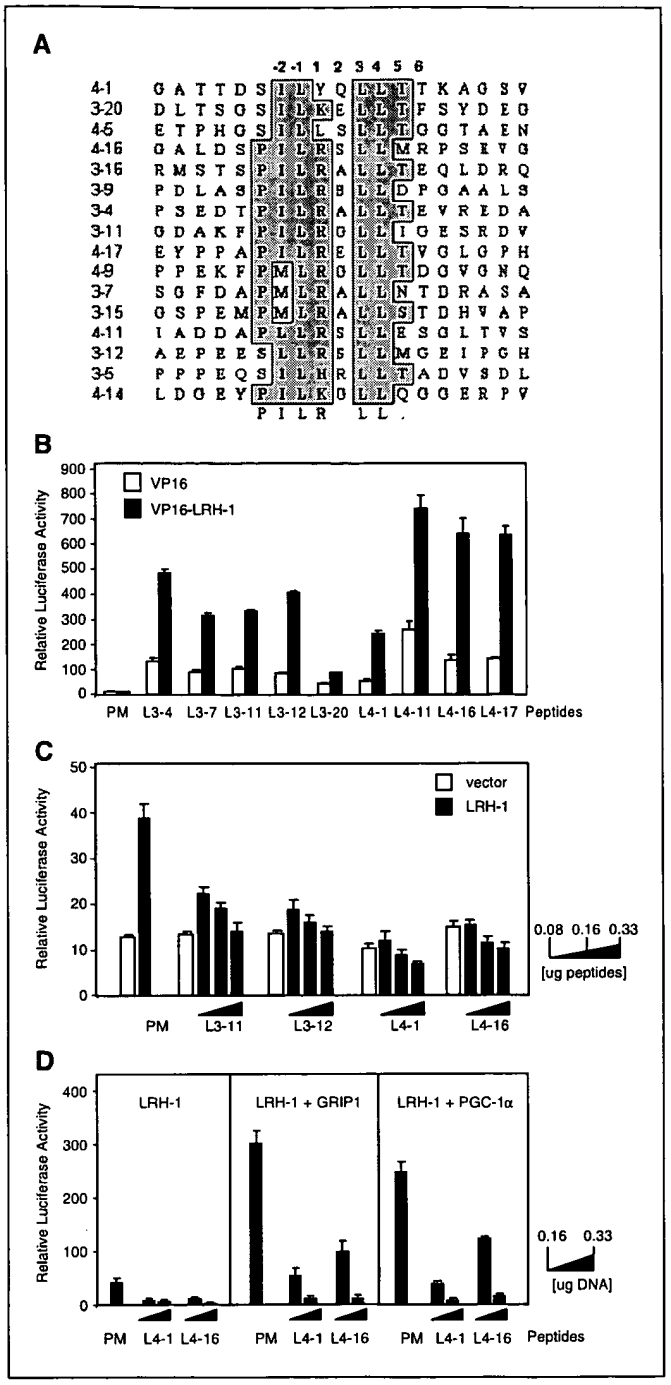

Figure 3. LRH-1-binding peptides interfere with its transcriptional activity. *A*, affinity selection of LRH-1-binding motifs using phage display technology. Baculovirus-expressed full-length LRH-1 was immobilized on DNA containing an SFRE in 96-well Costar plates as a screening target. Phage that interacted specifically with LRH-1 were selected, and the peptide sequences were deduced by DNA sequencing. *B*, Gal4-LXXLL peptide fusion interacts with LRH-1 in mammalian two-hybrid assay. HepG2 cells were cotransfected with CMVβ-Gal, either the empty pM vector or the indicated fusion peptides, and either the empty VP16 vector or VP16-LRH-1. *C*, Gal4-DBD fusion peptides suppress LRH-1-stimulated transcription from a synthetic SFRE. HeLa cells were cotransfected with a consensus SFRE promoter 5xSFRE-TATA-Luc reporter gene and increasing amounts of the Gal4-DBD-peptide fusion constructs (as indicated) in the presence (*solid columns*) or absence (*open columns*) of an LRH-1 expression plasmid. *D*, LRH-1 peptides disrupt GRIP1 and PGC-1α coactivation of LRH-1. HeLa cells were cotransfected with an aromatase promoter II-luciferase reporter construct, LRH-1 expression plasmid in the presence or absence of GRIP1 or PGC-1α, and increasing concentrations of the Gal4-DBD-peptide fusion constructs.

that in the absence of any known ligands, LRH-1 seems to exist in a conformation that is capable of binding the LXXLL motifs present in coactivators. Furthermore, these affinity-selected peptides efficiently block LRH-1-mediated transcriptional activity by competitively inhibiting the interaction of the receptor with required cofactors.

LRH-1 peptides disrupt GRIP1 and PGC-1α coactivation of LRH-1. The peptide antagonists L4-1 and L4-16 effectively repressed LRH-1 transcriptional activity on a simple response element SFRE within intact cells (Fig. 3*C*). We next assessed the ability of these peptides to inhibit LRH-1 transcriptional activity on the more relevant aromatase promoter II in the presence or absence of coexpressed PGC-1α or GRIP1. HeLa cells were cotransfected with the aromatase promoter II reporter and an LRH-1 expression vector in the presence of either pM (encoding the Gal4-DBD) or increasing amounts of the Gal4-DBD fusion peptides. As expected, L4-1 and L4-16 inhibited both the basal and coactivator-induced LRH-1 transcriptional activity on the aromatase promoter II (Fig. 3*D*). Together, these data show that the peptides can repress LRH-1 activity on a complex promoter by competitively inhibiting LRH-1/coactivator interactions.

All LRH-1–binding peptides also interact with retinoid X receptor α. To address the specificity of the LRH-1-interacting peptides identified in this screen, we assessed their ability to bind an RXRα-VP16 fusion protein in a mammalian two-hybrid assay. Interestingly, all of the LRH-1-interacting peptides identified also interacted with activated RXR bound to 9-*cis*-retinoic acid (9-*cis*-RA) or the selective RXR agonist LG100268 (Fig. 4). Treatment with the RXR antagonist LG101208 abolished both the basal and agonist-induced interaction of the peptides with RXR, indicating that they probably also interact with the canonical coactivator binding surface on this receptor (Fig. 4). These results suggest that activated RXR shares identical or highly similar surfaces with LRH-1, raising the possibility that LRH-1 and RXR can be activated by similar coregulators within target cells.

Retinoid X receptor agonists inhibit LRH-1–mediated transcription. Given that LRH-1 peptide antagonists also interact with RXR, it is likely that activation of RXR has effects on LRH-1 signaling within target cells. More specifically, we hypothesized that RXR ligands might inhibit LRH-1-mediated transcriptional activity by enabling activated RXRα to compete with LRH-1 for common coactivators (i.e., antagonism independent of DNA binding). To address this possibility, we transfected HeLa cells with LRH-1 and RXRα expression vectors, and a luciferase reporter gene construct containing five copies of the SFRE. As shown in Fig. 5*A*, LRH-1 stimulated the SFRE reporter by ~3-fold. Addition of increasing concentrations of 9-*cis*-RA inhibited LRH-1-mediated transcription in a concentration-dependent manner, with complete inhibition occurring at 0.1 μmol/L of 9-*cis*-RA. Therefore, activated RXR can completely block LRH-1-mediated transcriptional activity, and this inhibition can occur on a promoter to which RXR does not bind in a direct manner. We next asked if LRH-1 inhibition by RXR is agonist dependent. 9-*cis*-RA and the selective RXR ligand LG100268 are full agonists and activate RXRα-mediated transcriptional activity on a DR1 response element. In contrast, LG101208 is a full antagonist, whereas LG101506 is a partial agonist (Fig. 5*B*). When the 5xSFRE reporter construct was cotransfected with LRH-1 and RXRα, 9-*cis*-RA as well as the selective RXR ligand LG100268 fully repressed LRH-1-mediated transcription, whereas the partial agonist LG101506 had a partial inhibitory effect (Fig. 5*C*). Significantly, the full-antagonist LG101208 completely

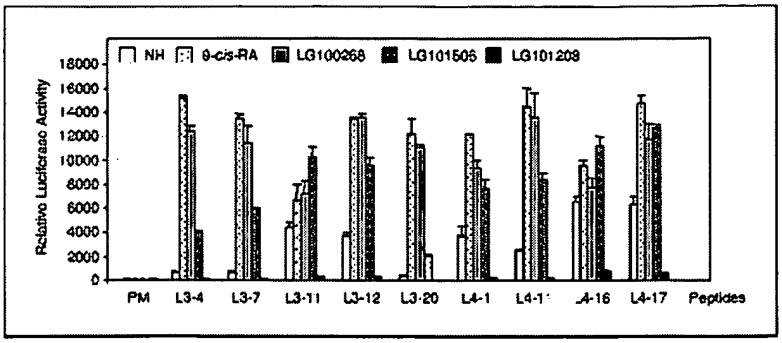

Figure 4. LRH-1–interacting peptides also interact with activated RXR. HepG2 cells were cotransfected with a 5xGal4-luciferase reporter construct, a Gal4-DBD-fusion peptide, and either an empty VP16 vector or VP16-tagged RXRα. Twenty-four hours following transfection, cells were treated overnight with either the full agonists 9-*cis*-RA or LG100268 at 0.1 μmol/L, the partial agonist LG101506 at 0.1 μmol/L, or the full antagonist LG101208 at 1 μmol/L.

reversed the inhibition of LRH-1 transcriptional activity by either 9-*cis*-RA or LG100268 (Fig. 5*C*). Together, these data indicate that inhibition of LRH-1 by RXR is agonist dependent, and RXR requires an active conformation to mediate this repression. Thus, LRH-1 inhibition by activated RXR may occur as a consequence of competition for common coregulators used by these two transcription factors.

To further show that this inhibition is through direct competition for coactivators used by both receptors, we designed a transfection experiment in which Gal4-LRH-1 LBD and RXRα were coexpressed in the presence or absence of 9-*cis*-RA, PGC-1α, or GRIP1. We used the Gal4 system to exclude the possibility that RXR might interfere with LRH-1 binding to its response element. As shown in Fig. 5*D*, Gal4-LRH-1 LBD activated transcription from the 5xGal4 response element reporter construct, and this activity was further enhanced in the presence of PGC-1α or GRIP1. In this context, 9-*cis*-RA strongly repressed basal activity of LRH-1 LBD (Fig. 5*E*). More interestingly, activated RXR repressed the stimulation of LRH-1 by either PGC-1α or GRIP1 (Fig. 5*E*). These results indicate that in the presence of 9-*cis*-RA, RXR is able to compete for coactivators present within the cell required for LRH-1 transcriptional activity.

9-*cis*-retinoic acid inhibits transcription from promoter II of the *CYP19* gene. Because LRH-1 is implicated as a key transcription factor mediating aromatase expression in breast tissue (7), we asked whether 9-*cis*-RA can repress LRH-1-mediated transcriptional activity on aromatase promoter II. The major inducer of aromatase expression in breast preadipocytes is prostaglandin E_2 (PGE_2) derived from breast tumor epithelium or macrophage infiltration (25). Here, we used forskolin and PMA to mimic PGE_2 signaling through protein kinases A and C (25). NIH-3T3 cells were transfected with an LRH-1 expression vector in the presence or absence of forskolin and PMA. In the absence of stimulation, LRH-1 produced a 10-fold increase in the transcriptional activity of promoter II (Fig. 6*A*). In the absence of exogenous LRH-1, forskolin and PMA treatment produced a 100-fold increase in the transcriptional activity of promoter II. In combination, however, LRH-1, forskolin, and PMA had a synergistic effect, increasing promoter II activity by 500-fold. Addition of 9-*cis*-RA repressed LRH-1-mediated transcription (10.9- to 2.9-fold). Moreover, 9-*cis*-RA was able to repress LRH-1, forskolin, and PMA synergism on promoter II (by 80%). Therefore, 9-*cis*-RA can inhibit hormone-induced aromatase promoter activity by inhibiting LRH-1 via sequestration of coactivators by endogenously activated RXR.

To confirm that this action results in inhibition of endogenous aromatase activity, human breast adipose stromal cells were incubated with forskolin plus PMA, together with the RXR ligand LG101305, in various concentrations (Fig. 6*B*). This ligand inhibited aromatase activity of the cells in a concentration-dependent fashion. Thus, the inhibitory activity of RXR agonists on the isolated aromatase promoter II is recapitulated on the endogenous gene in the absence of ectopic receptor expression.

Discussion

LRH-1 regulates diverse processes, including endodermal development, reverse cholesterol transport, bile acid homeostasis, and steroidogenesis (3). No natural agonists for LRH-1 have yet been identified, although the crystallized ligand binding domains of this receptor were found to contain phospholipids. The biological significance of these observations remains to be determined (12). It seems more likely that LRH-1 transcriptional activity is regulated by other means (e.g., by post-translational modification, heterodimer interactions with NR0B family members, or by the nature and availability of coregulator proteins). Here, we show novel interactions between LRH-1 and PGC-1α that have powerful effects on LRH-1 transcriptional activity and, more importantly, are amenable to modulation by small molecules, either positively via cAMP-induced PGC-1α expression, or negatively via rexinoid-induced RXR activation and consequent sequestration of endogenous LRH-1 coactivators.

PGC-1α is an LRH-1 coactivator. Previous studies have suggested that LRH-1 exhibits weak binding to coregulators and indeed displays little discrimination between coactivators, such as SRC-1 and corepressors, such as SMRT (11). We show here that PGC-1α (but not PGC-1β) is a strong coactivator of LRH-1 in the context of aromatase promoter II through direct interaction via its consensus LXXLL box. The significance of this lies in the fact that unlike many other coregulators that are ubiquitously expressed and (in general) not hormonally regulated, PGC-1α exhibits marked tissue-specific expression that can be strongly regulated by cAMP. In mice, PGC-1α is expressed predominantly in brown adipose tissue, heart, kidney, and brain (18), whereas in humans, it is also expressed in white adipose tissue (26). Although expression in white adipose tissue is relatively low, we show here that PGC-1α mRNA levels in human breast preadipocytes can be markedly increased by cAMP treatment. This confirms that PGC-1α expression is regulated by cAMP in white adipose tissue, as it is in brown adipose tissue (27) and liver (28). More importantly, PGC-1α thus provides a mechanism whereby hormonal signals, via cAMP, could regulate LRH-1 transcriptional activity in the absence of any cognate ligand. The consequences of this for LRH-1 target gene expression are illustrated by the marked stimulation of both PGC-1α and aromatase mRNA expression in preadipocytes in response to forskolin (Fig. 2).

LRH-1 as a target for drug discovery in breast cancer. Although LRH-1 has been implicated in a variety of physiological processes, such as bile acid and cholesterol homeostasis (3), it is not yet clear whether modulation of its transcriptional activity will have therapeutic value. However, its role in regulating estrogen production in breast tissue presents a unique opportunity to develop more specific antiestrogen therapies for breast cancer. Adjuvant hormonal therapy for estrogen-dependent breast cancer targets the mitogenic actions of estrogen by blocking either its action at the level of the estrogen receptor (ER) or its synthesis by aromatase (29). Current phase 3 inhibitors of aromatase catalytic activity are proving superior to the ER antagonist tamoxifen in both primary and secondary end points (30, 31). However, these agents block estrogen formation in all tissues where aromatase is expressed, not only in breast, thus preventing estrogen's beneficial agonist effects in bone, brain, and the cardiovascular system. Future strategies should therefore be aimed at developing drugs that will inhibit estrogen production in sites where estrogen may promote growth of transformed cells, such as the breast, and not in tissues where estrogen action is favorable, such as the bone and brain. This can only be achieved by inhibiting aromatase expression in a tissue-specific fashion (i.e., by the development of selective aromatase modulators). The promoter used to direct aromatase expression in breast tumors, promoter II (and its overlapping partner, promoter I.3), is different from that used in any other tissue of postmenopausal women due to activation by tumor-derived PGE_2 (25). It follows that a major regulator of promoter II expression in breast, LRH-1, is a potential target for the development of such selective aromatase modulators (reviewed in ref. 32).

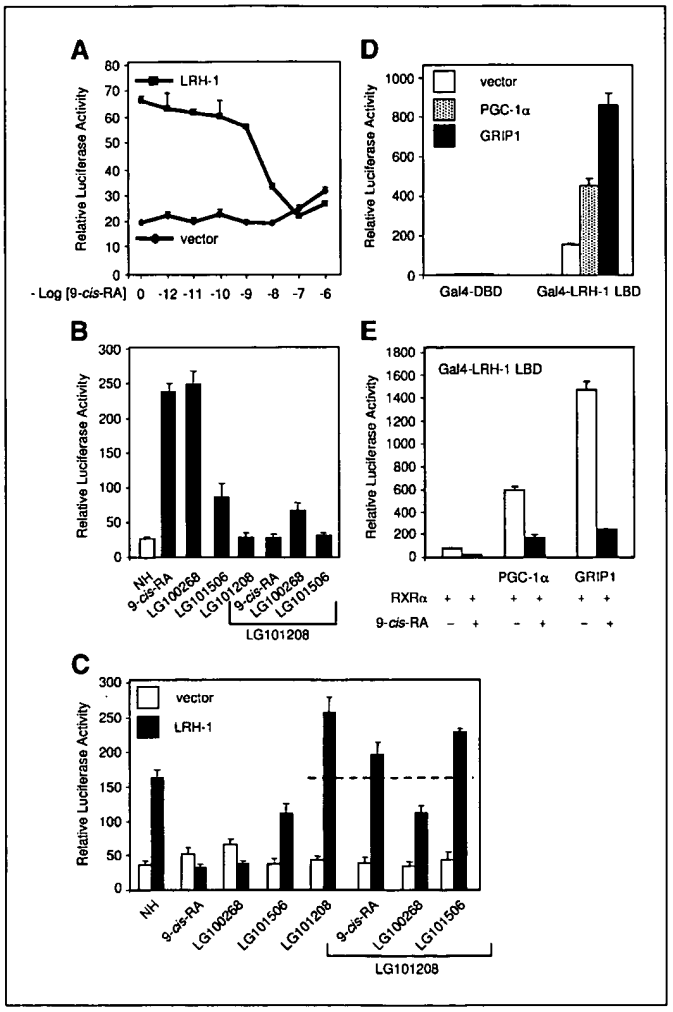

Figure 5. RXR agonists inhibit LRH-1-mediated transcription. *A*, 9-*cis*-RA-activated RXRα represses LRH-1 on a synthetic SFRE. HeLa cells were cotransfected with a 5xSFRE-TATA-luciferase reporter construct and LRH-1 and RXRα expression vectors in the absence or presence of increasing concentration of 9-*cis*-RA. *B*, RXRα transcriptional activity with different ligands. HeLa cells were cotransfected with a DR1 response element-luciferase reporter construct and RXRα expression vector in the presence or absence of different RXR ligands. *C*, only RXRα agonists inhibit LRH-1 transcriptional activity. HeLa cells were cotransfected with a 5xSFRE-TATA-luciferase reporter construct and LRH-1 and RXRα expression vectors in the absence or presence of different RXR ligands. *D*, GRIP1 and PGC-1α coactivate Gal4-LRH-1 LBD fusion protein. HeLa cells were cotransfected with a 5xGal4-luciferase reporter construct and GaL4-LRH-1 LBD in the absence or presence of GRIP1 or PGC-1α. *E*, activated RXR represses GRIP1 and PGC-1α coactivation of LRH-1. HeLa cells were cotransfected with a 5xGal4-luciferase reporter construct and Gal4-LRH-1 LBD in the presence or absence of GRIP1 and PGC-1α and RXRα expression vector in the presence or absence of 9-*cis*-RA.

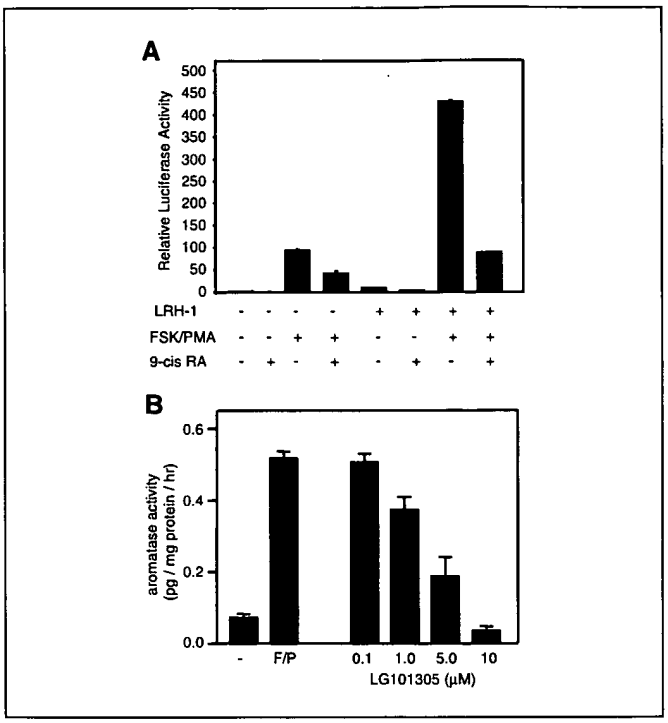

Figure 6. 9-*cis*-RA inhibits aromatase transcription from promoter II. *A*, NIH-3T3 cells were cotransfected with an aromatase promoter II-luciferase reporter construct (PII-516) and LRH-1 expression vector in the presence or absence of 25 μmol/L forskolin (*FSK*), 4 nmol/L PMA, and 9-*cis*-RA. *B*, primary human breast adipose stromal cells were treated with or without forskolin (25 μmol/L)/PMA (4 nmol/L), in the presence of the indicated concentrations of the RXR agonist LG101305 (*LG*). Twenty-four hours later, aromatase activity was measured. *Columns*, means of triplicate determinations from one representative experiment; *bars*, SD. Similar results were obtained in two further independent experiments.

Inhibition of LRH-1 transcriptional activity. To date, the only inhibitors of LRH-1 activity identified have been protein partners, such as SHP (21–23), DAX-1 (24), and Prox1 (33–35). In this study, we have identified several small LXXLL-containing peptide antagonists of LRH-1, all of which belong to the class III-like LXXLL peptide family, and act by disrupting LRH-1/coactivator interactions. Given the state of the art in the field of peptidomimetic drug design, it is possible that derivatives of the LRH-1-interacting

peptides could be created, which directly interfere with the function of the AF-2 pocket of the receptor. However, our data showing that RXR and LRH-1 are likely to be regulated by similar endogenous coactivators has highlighted a more near-term clinical opportunity for a breast cancer therapeutic. Specifically, we show that 9-*cis*-RA inhibits PGC-1α and GRIP1-induced LRH-1 activity and potently inhibits (by up to 80%) aromatase promoter activity induced by LRH-1 and forskolin. This again provides a mechanism for the modulation, in this case, inhibition, of LRH-1 transcriptional activity and expression of LRH-1 target genes by small molecules and illustrates a level of crosstalk between the RXR and LRH-1 pathways.

RXR ligands are promising molecules for both chemoprevention and therapy of cancer (36). In particular, 9-*cis*-RA and RXR-selective ligands are highly efficacious in preventing breast cancer in experimental animal models (37, 38). The mechanisms by which rexinoids suppress carcinogenesis are not fully understood, in part due to their large spectra of action. However, it is likely that inhibition of local estrogen synthesis by aromatase in breast preadipocytes contributes to their efficacy. Thiazolidinediones, which are PPARγ ligands, have also been shown to inhibit proliferation of breast cancer cells (39). We previously showed that RXR and PPARγ ligands inhibit aromatase expression from promoter II and promoter I.4 in primary human preadipocytes via an indirect mechanism that does not involve binding of either RXR or PPARγ to the aromatase promoter (40). One mechanism by which rexinoids and PPARγ ligands could exert antiproliferative effects in ER-positive breast cancers is the sequesteration of LRH-1 coactivators by activated RXR with resulting inhibition of LRH-1-induced local aromatase expression and estrogen production. However, rexinoids are also effective growth inhibitor agents in ER-negative tumors (38). We have observed an increase in the proliferation rate of MCF-7 breast cancer cells that modestly (3-fold) overexpress LRH-1, associated with increased expression of cyclin D1,[4] suggesting that LRH-1 directly controls cell cycle progression in breast cancer cells, as it does in colon cancer (41). Thus, we speculate that rexinoids may in part inhibit breast cancer cell proliferation through inhibiting LRH-1 transcriptional activity and cell cycle progression.

In summary, we have identified novel hormone-regulated coregulator interactions that modulate LRH-1 transcriptional activity. Because deletion of LRH-1 results in embryonic lethality in mice (42), knowledge of the signaling pathways that modulate its activity, coupled with the development of highly specific peptide antagonists of LRH-1 as described here, will greatly facilitate understanding of the roles of LRH-1 in health and disease and validate LRH-1 as a therapeutic target. In particular, the identification of small molecules, such as rexinoids that inhibit LRH-1 activation of aromatase in breast, suggests that such molecules fulfill the criteria of selective aromatase modulators, and that LRH-1 could be a target for breast-specific tumor therapy.

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References

1. Lavorgna G, Ueda H, Clos J, Wu C. FTZ-F1, a steroid hormone receptor-like protein implicated in the activation of fushi tarazu. Science 1991;252:848–51.
2. Parker KL, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 1997;18:361–77.
3. Fayard E, Auwerx J, Schoonjans K. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends Cell Biol 2004;14:250–60.
4. Falender AE, Lanz R, Malenfant D, Belanger L, Richards JS. Differential expression of steroidogenic factor-1 and FTF/LRH-1 in the rodent ovary. Endocrinology 2003;144:3598–610.
5. Liu DL, Liu WZ, Li QL, et al. Expression and functional analysis of liver receptor homologue 1 as a potential steroidogenic factor in rat ovary. Biol Reprod 2003;69:508–17.
6. Pezzi V, Sirianni R, Chimento A, et al. Differential expression of SF-1/AD4BP and LRH-1/FTF in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. Endocrinology 2004;145:2186–96.
7. Clyne CD, Speed CJ, Zhou J, Simpson ER. Liver receptor homologue-1 (LRH-1) regulates expression of aromatase in preadipocytes. J Biol Chem 2002;277:20591–7.
8. Zhou J, Suzuki T, Kovacic A, et al. Interactions between prostaglandin E_2, liver receptor homologue-1 (LRH-1) and aromatase in breast cancer. Cancer Res 2005;65:657–63.
9. Agarwal VR, Bulun SE, Leitch M, Rohrich R, Simpson ER. Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients. J Clin Endocrinol Metab 1996;81:3843–9.
10. McKenna NJ, O'Malley BW. Minireview: nuclear receptor coactivators—an update. Endocrinology 2002;143:2461–5.
11. Sablin EP, Krylova IN, Fletterick RJ, Ingraham HA. Structural basis for ligand-independent activation of the orphan nuclear receptor LRH-1. Mol Cell 2003;11:1575–85.
12. Ortlund EA, Lee Y, Solomon IH, et al. Modulation of human nuclear receptor LRH-1 activity by phospholipids and SHP. Nat Struct Mol Biol 2005;12:357–63.
13. Krylova IN, Sablin EP, Moore J, et al. Structural analyses reveal phosphatidyl inositols as ligands for the NR5 orphan receptors SF-1 and LRH-1. Cell 2005;120:343–55.
14. Wang W, Zhang C, Marimuthu A, et al. The crystal structures of human steroidogenic factor-1 and liver receptor homologue-1. Proc Natl Acad Sci U S A 2005;102:7505–10.
15. Chang C, Norris JD, Gron H, et al. Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors α and β. Mol Cell Biol 1999;19:8226–39.
16. Ackerman GE, Smith ME, Mendelson CR, MacDonald PC, Simpson ER. Aromatization of androstenedione by human adipose tissue stromal cells in monolayer culture. J Clin Endocrinol Metab 1981;53:412–7.
17. Lee YK, Parker KL, Choi HS, Moore DD. Activation of the promoter of the orphan receptor SHP by orphan receptors that bind DNA as monomers. J Biol Chem 1999;274:20869–73.
18. Puigserver P, Wu Z, Park CW, et al. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 1998;92:829–39.
19. Lin J, Puigserver P, Donovan J, Tarr P, Spiegelman BM. Peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β), a novel PGC-1-related transcription coactivator associated with host cell factor. J Biol Chem 2002;277:1645–8.
20. Knutti D, Kaul A, Kralli A. A tissue-specific coactivator of steroid receptors, identified in a functional genetic screen. Mol Cell Biol 2000;20:2411–22.
21. Kovacic A, Speed CJ, Simpson ER, Clyne CD. Inhibition of aromatase transcription via promoter II by short heterodimer partner (SHP) in human preadipocytes. Mol Endocrinol 2004;18:252–9.
22. Goodwin B, Jones SA, Price RR, et al. A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. Mol Cell 2000;6:517–26.
23. Lu TT, Makishima M, Repa JJ, et al. Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. Mol Cell 2000;6:507–15.
24. Suzuki T, Kasahara M, Yoshioka H, Morohashi K, Umesono K. LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1. Mol Cell Biol 2003;23:238–49.
25. Zhao Y, Agarwal VR, Mendelson CR, Simpson ER. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology 1996;137:5739–42.
26. Larrouy D, Vidal H, Andreelli F, Laville M, Langin D. Cloning and mRNA tissue distribution of human PPARγ coactivator-1. Int J Obes Relat Metab Disord 1999;23:1327–32.

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27. Cao W, Daniel KW, Robidoux J, et al. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol Cell Biol 2004;24:3057–67.
28. Herzig S, Long F, Jhala US, et al. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 2001;413:179–83.
29. Santen RJ. To block estrogen's synthesis or action: that is the question. J Clin Endocrinol Metab 2002;87:3007–12.
30. Baum M, Budzar AU, Cuzick J, et al. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet 2002;359:2131–9.
31. Buzdar AU. Data from the Arimidex, tamoxifen, alone or in combination (ATAC) trial: implications for use of aromatase inhibitors in 2003. Clin Cancer Res 2004;10:355–61S.
32. Clyne CD, Kovacic A, Speed CJ, et al. Regulation of aromatase expression by the nuclear receptor LRH-1 in adipose tissue. Mol Cell Endocrinol 2004;215:39–44.
33. Liu YW, Gao W, Teh HL, Tan JH, Chan WK. Prox1 is a novel coregulator of Ff1b and is involved in the embryonic development of the zebra fish interrenal primordium. Mol Cell Biol 2003;23:7243–55.
34. Qin J, Gao DM, Jiang QF, et al. Prox1 is a corepressor of human liver receptor homologue-1 and suppresses the transcription of the cholesterol 7-α-hydroxylase gene. Mol Endocrinol 2004;18:2424–39.
35. Steffensen KR, Holter E, Bavner A, et al. Functional conservation of interactions between a homeodomain cofactor and a mammalian FTZ-F1 homologue. EMBO Rep 2004;5:613–9.
36. Lippman SM, Lotan R. Advances in the development of retinoids as chemopreventive agents. J Nutr 2000;130:479–82S.
37. Gottardis MM, Bischoff ED, Shirley MA, et al. Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. Cancer Res 1996;56:5566–70.
38. Wu K, Zhang Y, Xu XC, et al. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. Cancer Res 2002;62:6376–80.
39. Fenner MH, Elstner E. Peroxisome proliferator-activated receptor-γ ligands for the treatment of breast cancer. Expert Opin Investig Drugs 2005;14:557–68.
40. Rubin GL, Duong JH, Clyne CD, et al. Ligands for the peroxisomal proliferator-activated receptor γ and the retinoid X receptor inhibit aromatase cytochrome P450 (CYP19) expression mediated by promoter II in human breast adipose. Endocrinology 2002;143:2863–71.
41. Botrugno OA, Fayard E, Annicotte JS, et al. Synergy between LRH-1 and β-catenin induces G_1 cyclin-mediated cell proliferation. Mol Cell 2004;15:499–509.
42. Pare JF, Malenfant D, Courtemanche C, et al. The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis, and regulated by a DR4 element. J Biol Chem 2004;279:21206–16.